Site-specific Determinants of the Oral Microbiome

Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction at The Ohio State University

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The Ohio State University

2021

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Abstract

Oral microbes are regarded as the principal cause of periodontitis. However, next-generation sequencing studies have demonstrated that the transition from health to disease is attributed to a shift in the global balance of the microflora rather than to specific pathogens. This highlights the importance of examining each patient's oral microbiome as prerequisite for assessing individual disease susceptibility and for the development of personalized therapies. The objective of this study is to observe the differences, if any, in the oral microbiome of individuals at different sites and to determine the site-specific factors that contribute to these differences. Site-specific subgingival plaque samples were collected at two time points five years apart from 303 individuals in both healthy and periodontally diseased sites as part of the Study of Health in Pomerania (SHIP) effort. The same site was sampled at both timepoints. DNA was isolated from these samples and the V1-V3 and V4-V5 regions of the 16S rRNA gene was sequenced on the MiSeq platform. The sequences were aligned against the Human Oral Microbiome Database (HOMD) using BLAST to identify species present in each sample. Alpha and beta diversity metrics were computed to investigate the site-specific differences in health and disease. Linear Discriminant Analysis (LDA) was used to explore the influence of body mass index (BMI), diabetes, and smoking on the site-specific differences of the diseased oral microbiome. The microbial profiles of two sites

within the same individual did not demonstrate significantly greater similarity than between two periodontally healthy individuals or two subjects with periodontitis ($p>0.05$, ANOSIM of Bray-Curtis Dissimilarity Index). LDA demonstrated that BMI, diabetes and smoking were significant drivers of this site-specific heterogeneity in diseased individuals, but not in healthy subjects.

Dedication

I would like to dedicate this thesis to my parents, Aaron and Leslie, for encouraging me to get involved in research and the constant love and support throughout my time at Ohio State.

Acknowledgments

I would like to thank the following people, without whom I would not have been able to complete this research. Dr. Kumar for her insight, guidance, and support that steered me through this project. Dr. Machiraju, who agreed to serve as my co-advisor. Dr. Shareef Dabdoub for his help with pre-processing sequence data. Dr. Khaled Altabtbaei for his guidance and help throughout the data analysis portion of this project. Aakrati Chaudhry for her help with DNA Isolation, pre-processing, and data analysis. All other members of the Kumar Lab, whose contributions made the completion of this project possible.

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Chapter 1. Introduction

Background and Motivation

Periodontology is a dental specialty concerned with the prevention and treatment of diseases affecting the tissues that anchor teeth to the maxilla and mandible. Periodontitis, one of these diseases, is estimated to affect almost 47% of adults in the United States[1]. When left untreated, this disease can lead to tooth loss, lower nutritional status, and problems with mastication and speech. The clinical community has come to a consensus that periodontitis does not affect every site in the mouth, and the progression and severity of the disease at affected sites is heterogeneous. One of the goals of periodontal treatment is to reduce the pathologically deepened subgingival sulcus to levels maintainable by home care. Consequently, periodontal therapies must be personalized to both the patient and each diseased site.

The etiology of periodontitis has been studied extensively, but few studies have focused on the factors that determine the site-specific characteristics of the disease. Historically, research on the pathology of periodontitis has focused on immune-inflammatory mediators and bacteria as the etiological agents. Most of these studies have utilized one of two approaches: aggregating data at the patient level or pooling samples from sites with disease. For example, most clinical studies report probing depths, attachment or plaque levels averaged for the whole mouth[2]. Similarly, studies on site-

specific microbial profiles have used aggregations[3-10]. However, there are a few principal issues with this practice. Aggregations lead to a significant loss of power due to the relatively lower number of aggregated values available for data analysis. Additionally, utilizing such values leads to a loss of nuance in the data. For instance, a high plaque score on a few teeth and many teeth with moderate plaque scores will have the same average value. Lastly, aggregations assume that each site is an independent variable. However, each site is influenced by tooth-level and patient-level factors and behaviors[11]. There are issues with pooling samples as well. Recent research utilizing targeted DNA and proteomic methods have shown that pooled samples are not representative of the site-specific nature of periodontitis[12,13]. In order to increase the precision of periodontal treatment, the treatment must be based on disease characteristics specific to each site rather than average disease characteristics of the whole mouth.

The influence of patient-level factors on oral microbiome stability is well-documented. These factors, such as smoking, diabetes, and BMI, exert an environmental pressure on the oral microbiome. Moreover, all three are considered risk factors for periodontitis. Since periodontitis is associated with the dysbiosis of the oral microbiome, it is necessary to catalogue the effects of these environmental perturbations on the microbial ecosystem. Previous studies have found distinct ways that each of these perturbations effect the microbiome in both community structure and membership. Smoking creates a pathogen-rich, commensal-poor microbial ecosystem in periodontally healthy individuals that resembles ecosystems associated with periodontitis[14,15]. Hyperglycemia creates a microbial ecosystem that enforces habitat filtering, favoring

organisms that thrive under glucose-rich, pro-oxidant, protein-rich, and anaerobic conditions[16]. Lastly, multiple oral probiotic taxa are significantly associated with obesity[17].

Research Hypotheses and Objective

Based on the review of literature, we hypothesized that subjects with periodontitis at both sites would display a unique oral microbial profile at each affected site, which would manifest as significant differences between the alpha and beta diversity of the sites. We also hypothesized that BMI, smoking status, and diabetes would influence this site-specific microbial heterogeneity.

Chapter 2. Methodology

Subject Recruitment

Subjects were recruited for this study as a part of the Study of Health in Pomerania (SHIP). From this effort, clinical periodontal data was available from 303 individuals at two time points five years apart[18]. Subjects also completed demographic and behavioral questionnaires, as well as medical and oral health history reports at both timepoints.

Sample Collection

Probing depth (PD), clinical attachment loss (CAL), and the presence of bleeding on probing (BOP) were measured at every tooth as part of the SHIP effort. PD is the distance from the gingival margin to the bottom of the periodontal pocket. CAL is a measure that quantifies the extent that the periodontal support surrounding a tooth has been destroyed. BOP measures whether or not the gingiva bleeds when probed. Site-specific subgingival plaque samples were collected by inserting sterile endodontic paper-points into the subgingival crevice at two sites with the deepest PD and separately stored. The same site was sampled at both timepoints.

Subject Selection

The periodontal data was used to classify each site in the mouth as periodontally healthy or diseased. This classification was based on PD, CAL and BOP of the site. A site with

PD of less than or equal to 3 mm, CAL less than 2mm and no BOP was classified as periodontally healthy. A site that did not meet these criteria was classified as diseased. Based on this, the 303 subjects were classified into a healthy group (subjects who were periodontally healthy at both sampled sites) and a disease group (subjects had periodontal disease at both sampled sites). Based on these criteria, we analyzed samples from 31 healthy individuals and 52 individuals with periodontitis over two timepoints.

Sequencing and Analysis Pipeline

DNA was isolated using the MagMAX™ Total Nucleic Acid Isolation Kit from 1212 site-specific samples. Subsequently, the V1-V3 and V4-V5 regions of the 16S rRNA gene were sequenced on the Illumina MiSeq platform for each sample. The primers used for sequencing were 5′- GTT TGA TCC TGG CTC AG- 3′ and 5′- GTA TTA CCG CGG CAG CTG GCA C-3′ for V1-V3, and for V4-V5. Two primers were used because each primer can detect a range of genera that the other cannot. Used in combination, the primers recover a broader range of the microbiome than is possible with a single primer. However, some genera are detected by both primers. To prevent overcounting, the number of sequences assigned to an Operational Taxonomic Unit (OTU) by both primers was reduced by half. Primer averaging was completed as described previously[19] using the implementation in the PhyloToAST software suite[20]. Cutadapt was used to find and remove adapter sequences, primers, and Poly-A tails from raw sequence data. As sequencing technologies produce reads closer to the 3'- and 5'- ends, the quality of reads decreases. Incorrect reads in these regions can have adverse effects on downstream

analyses. Sickle was used to determine at what base to trim the 3'- and 5'- ends via a sliding window with quality and length thresholds. Illumina MiSeq generates reads from both ends of DNA fragments. PEAR was used to merge these reads to create a sequence with greater overall read length. V4-V5 sequences were preprocessed through Cutadapt, Sickle, and PEAR; while, V1-V3 sequences were preprocessed solely through Cutadapt and PEAR. Next, split_libraries.py was used in QIIME to split sequence data based on barcodes that corresponded to each sample. Lastly, pick_OTUs.py in QIIME was used to assign OTUs to sequences from each sample. The blast method was used to cluster OTUs by aligning sequences against the Human Oral Microbiome Database (HOMD). Analyses were completed using PhyloToAST and QIIME[20,21] pipelines.

Statistical Analysis

Principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity indices was used to investigate the clustering based on site and Adonis test was used to determine statistical significance of beta diversity differences based on site[22]. Linear discriminant analysis (LDA) was performed using PhyloToAST[20]. LDA clustering for BMI, diabetes status, and smoking status groups were conducted to explore the level of site-specific difference within different groups of these factors. Tukey's HSD and Dunn's method for joint ranking were used to test the significance of intragroup and intergroup LDA clustering differences based on site in JMP. Alpha diversity of s-OTUs was quantified using Abundance Coverage Estimator (ACE) and Shannon Evenness Index (Shannon) in

PhyloToAST. Significance was evaluated using Wilcoxon Rank Sum test. Results were visualized using PhyloToAST[19] as PCoA plots, kernel density plots, and LDA plots.

Chapter 3. Results

Clinical and Demographic Characteristics of Groups

Table 1 shows the demographic and clinical characteristics of the groups. A total of

Table 1 Clinical and Demographic Features of Subjects

	Subjects With Periodontitis (n=52)	Healthy Subjects (n=31)
Age	60 ± 12.7	41 ± 9.2
Sex (% male)	56%	26%
Diabetes Status (% diabetic)	17%	0%
BMI (underweight: normal weight: overweight: obese)	0:9:23:20	2:18:9:2
Smoking Status (% smokers)	27%	35%
Mean PD (mm)	4.79 ± 1.13	2.29 ± 0.64
Abbreviation: BMI, body mass index; PD, probe depth.		

Table 1 Clinical and Demographic Features of Subjects

2.7 million high quality, classifiable sequences from 166 samples were used for analysis.

Site-specificity of the Oral Microbiome in Health

In subjects that were periodontally healthy at both sites, principal coordinate analysis of Bray-Curtis dissimilarity indices did not show greater significant clustering of sites for V1-V3 or V4-V5 sequence regions (Adonis test, p = 0.75 & 0.18, respectively) within the same individual than between individuals (Figure 1, panels a-b). However, this difference could not be attributed to differences in alpha diversity (Figure 1c/e, p=0.24 & 0.16, Ace and Shannon indices computed on the V1-V3 region profiles respectively). The same lack of significance was observed for the Shannon diversity metric in the V4-V5 region sequences (Figure 1f, P=0.16). However, there was a significant difference in Ace between different sites within the same individual in the V4-V5 region (Figure 1d, P=0.01). Kernel density plots of the previous alpha diversity metrics can be seen in

Figures 1c-f. The peaks indicate the median values for each group, and the x-axis shows the data range.

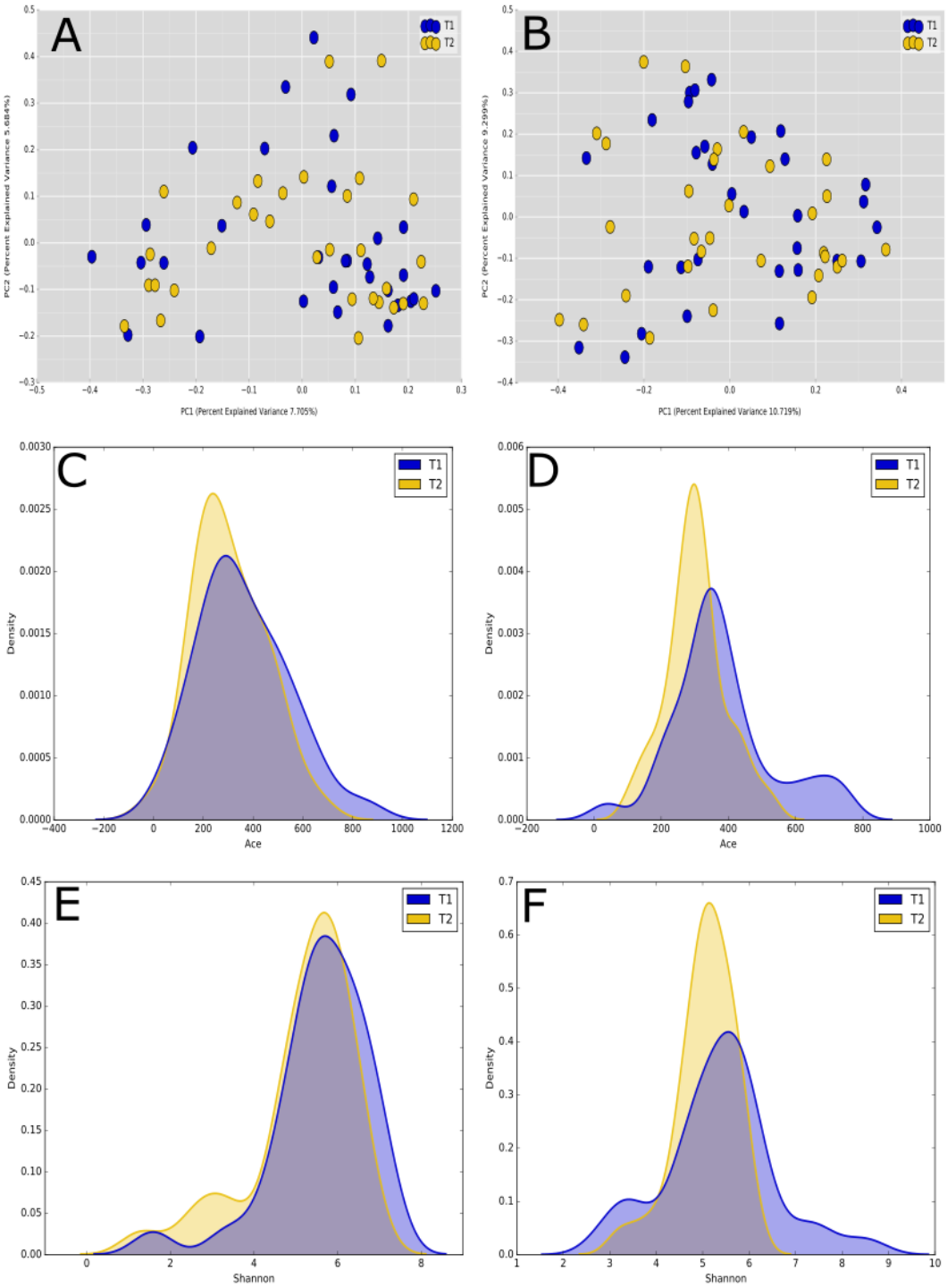

Figure 1 Alpha and Beta Diversity in Healthy Subjects
(a-b) show the principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity indices between two sites within the same individual in the V1-V3 and V4-V5 regions, respectively.**(c-f)** show the kernel density plots of alpha diversity (ace in a,b and Shannon in c,d) between two different sites in the same individual for the V1-V3 (a,c) and V4-V5 (b,d) sequences. The peak indicates the median values for each group and the x axis shows the data range. Only the ace values of the V4-V5 region differed significantly between two sites in the same individual.

Site-specificity of Oral Microbiome in Disease

Similar to healthy subjects, in subjects with periodontitis at both sites, sites within the same individual did not exhibit greater similarity than those between subjects (P=0.87 & 0.64, Bray-Curtis similarity Index of V1-V3 or V4-V5 sequence regions respectively, Figure 2a-b). However, as with healthy sites, this heterogeneity was not based on differences in alpha diversity (P=0.67 & 0.96, Ace and Shannon in the V1-V3 region sequences respectively, and P=0.24 & 0.73 V4-V5 region sequences). Kernel density plots of the previous alpha diversity metrics can be seen in Figures 2c-f.

Drivers of Site-specificity of in Disease

Linear discriminant analysis (LDA) revealed significant clustering of microbial profiles based on both BMI and periodontal health. Figures 3a-b show the LDA of BMI and periodontal health in diseased and healthy subjects, respectively. Figure 3b shows that healthy individuals contain sites that are microbially homogenous based on beta diversity as indicated by the lack of difference between groups of the same BMI at different sites except for the underweight group (P=0.03). Figure 3a shows an increased influence of BMI on the microbially profiles at each site, suggested by the significant difference between sites in the normal BMI individuals (P=0.02) and obese individuals (P=0.01). Also, significant clustering was observed based on smoking status between two sites within a single individual. Figure 3d does not show a significant clustering based on smoking status in periodontally healthy individuals. However, Figure 3c shows a significant difference between sites in smokers with periodontitis at both sites

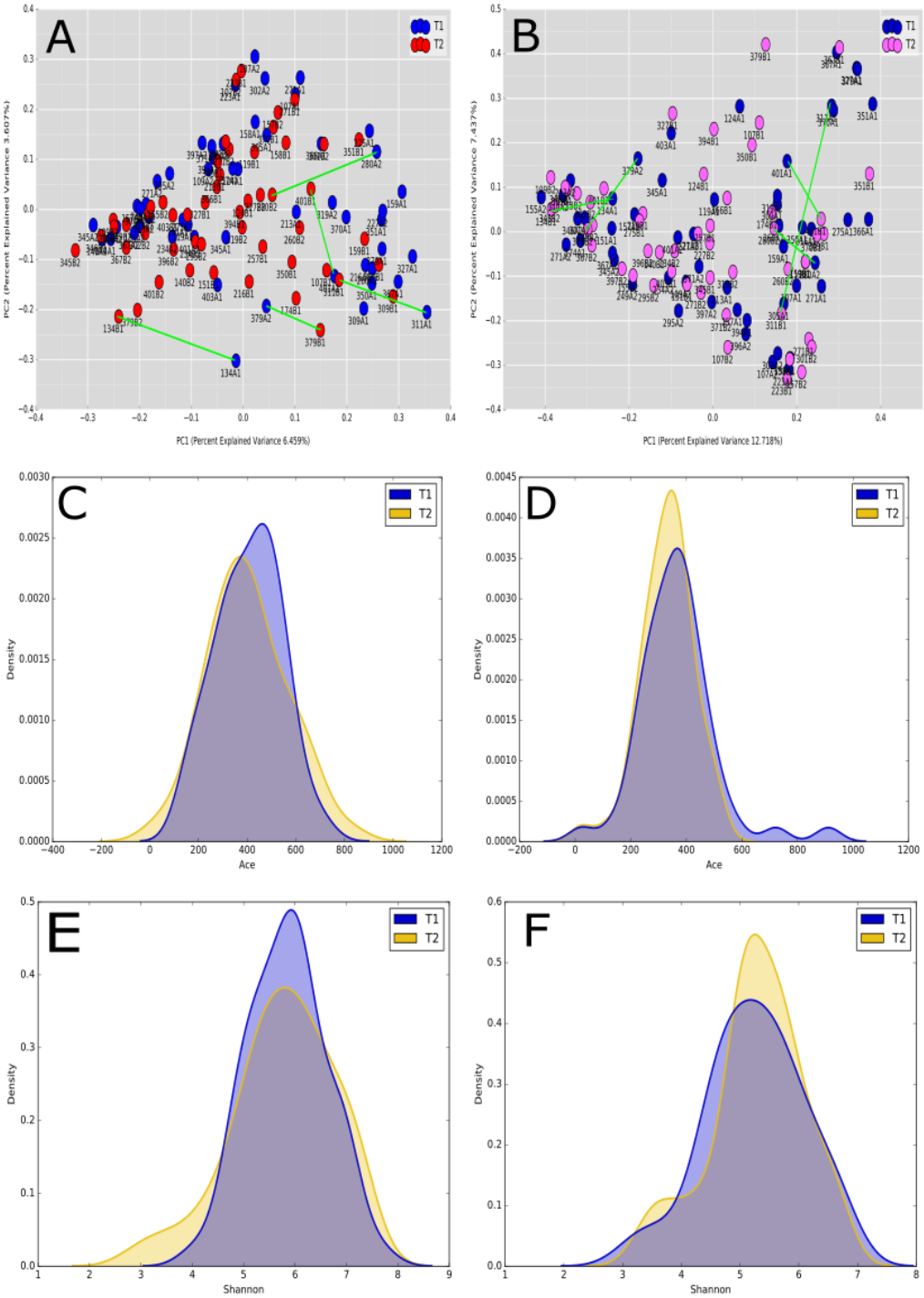

Figure 2 Alpha and Beta Diversity in Disease Subjects
(a-b) show the principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity indices between two sites within the same individual in the V1-V3 and V4-V5 regions, respectively. Green lines demonstrate the distance between two sites in the same individual based on their microbial similarity. (**c-f)** show the kernel density plots of alpha diversity (ace in a,b and Shannon in c,d) between two different sites in the same individual for the V1-V3 (a,c) and V4-V5 (b,d) sequences. The peak indicates the median values for each group and the x axis shows the data range.

(P=0.0015). Lastly, significant clustering was observed based on diabetes status between two sites within the same individual who had periodontitis at two sites. Figure 3e shows a significant difference between the microbial profile of two sites in nondiabetics with periodontitis at both sites (P<0.0001).

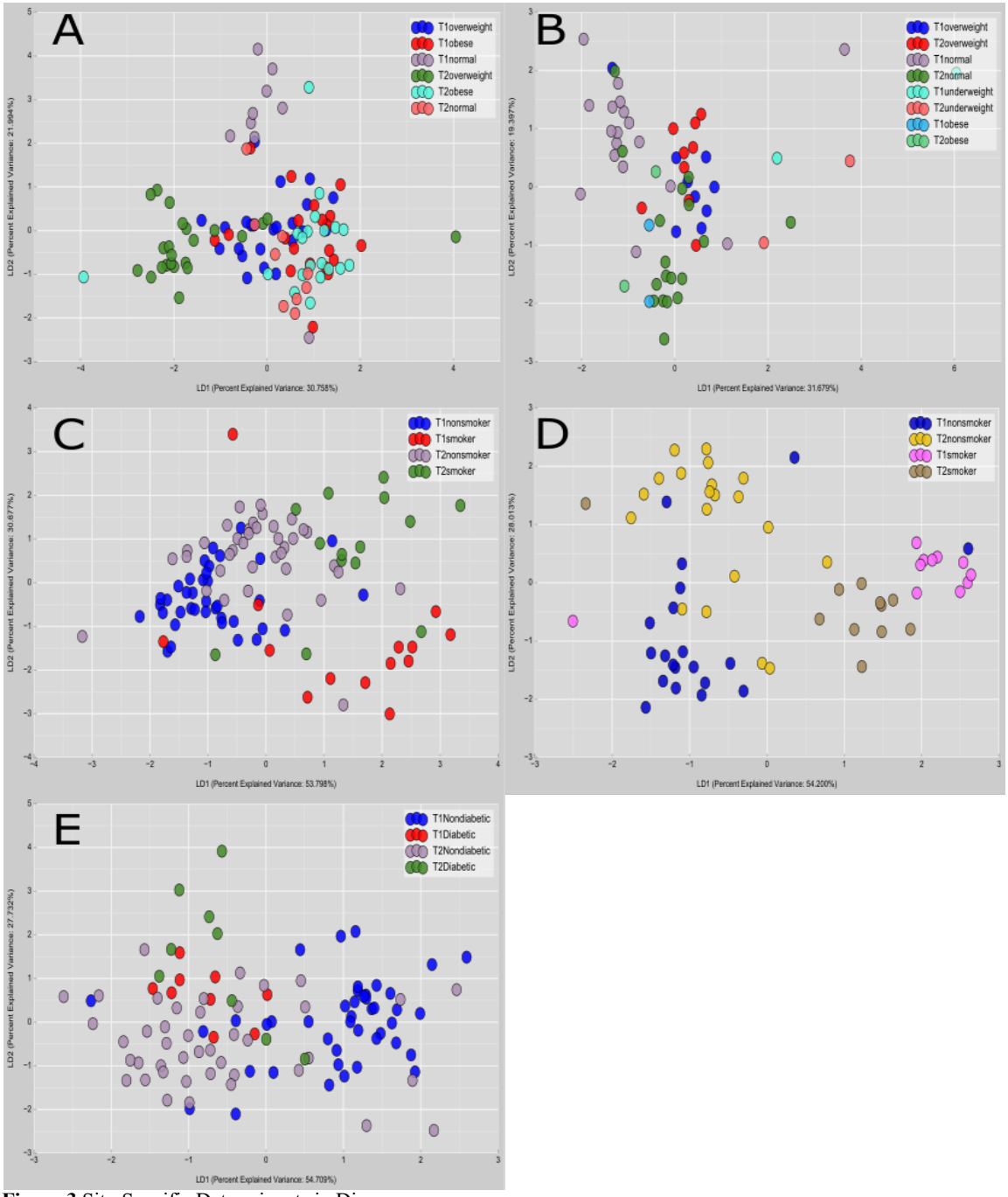

Figure 3 Site-Specific Determinants in Disease

Linear Discriminant Analysis (LDA) of species-level operational taxonomic units (s-OTUs) in periodontally healthy individuals who are smokers, nonsmokers, normal weight, underweight, overweight, and obese and individuals with periodontitis who are smokers, nonsmokers, normal weight, overweight, obese, diabetic, and nondiabetic. **(a)** Clustering of BMI groups in individuals with periodontitis. Significant intragroup difference based on site in normal (P=0.02) and obese (P=0.01) groups. **(b)** Clustering of BMI groups in healthy individuals. **(c)** Clustering of smoking status groups in individuals with periodontitis. Significant intragroup difference based on site in smoker (P=0.0015) group. **(d)** Clustering of smoking status groups in healthy individuals. **(e)** Clustering of diabetes status groups in individuals with periodontitis. Significant intragroup difference based on site in nondiabetic (P<0.0001) group.

Chapter 4. Discussion

Periodontitis is a site-specific disease, with the disease affecting many, but not all teeth in an individual with disease. Also, disease progression occurs through recurrent bursts of destruction followed by varying periods of quiescence at any given site. This temporally and spatially haphazard mode of disease progression has been named the random burst model, and therefore, the behavior of each site over time is the only measurable estimate of disease progression as well as therapeutic outcomes. In this study, we demonstrate that each site in the mouth harbors a unique microbiome, irrespective of whether the sites are healthy or have periodontitis. Our results corroborate previous studies in the literature that have examined the biogeography of the oral microbiome in health and dental caries[23,24] and advance the field forward by demonstrating similar site-specific differences in periodontitis. Our data serves to explain the random burst model of disease progression.

Previous studies have demonstrated that a gradient exists in the types of bacteria that colonize each tooth site, and that this gradient is based on the direction of salivary flow. We were unable to identify similar gradients in our study since we only sampled two sites per individual. Instead, we examined the effects of patient-level factors on site-specific microbial heterogeneity, notably those factors that are known to increase

susceptibility to periodontitis. We discovered that BMI, smoking and hyperglycemia significantly influence the degree of dissimilarity between microbiota in each site within an individual.

Importantly, we found that these differences between sites within the same mouth can be attributed to differing levels of the same species rather than differences in the types of species.

The connection between patient-level risk factors and periodontitis have been studied extensively. Obesity is identified as a risk factor for periodontitis[25]. Also, obesity's influence on the oral microbiome at the mouth-level has been investigated. A BMI greater than 30 is associated with a dysbiotic oral microbiome[26]. Since obese subjects appeared to have a different beta diversity compared to normal BMI subjects, it suggests that a large BMI is associated with a dysbiotic oral microbiome at the site-level as well. Also, the lack of influence of BMI on the beta diversity of healthy subjects compared to the distinct groups of beta diversity seen at each site based on BMI, suggest that BMI is contributing to differences in the microbiome among diseased sites. Previous studies have found that the dysbiotic shifts are correlated with subgingival adipokine levels and increased levels of pro-inflammatory cytokines in the gingival crevicular fluid[25]. Future work should determine how the influence of these cytokines and adipokines could lead to distinct microbiomes at different sites within the same individual.

Smoking's influence on periodontitis risk is well-known as well. It is a risk factor for the initiation, extent, and severity of periodontitis[27]. The influence of smoking on the

oral microbiome at the mouth level has been studied as well. The subgingival environment in smokers is anaerobic, acidic, reducing, nutritionally deprived, and immune-impaired. This leads to a pathogen-rich, commensal-poor microbial ecosystem[16]. The differences in beta diversity among sites in subjects with periodontitis compared to healthy subjects' sites suggests that the global changes seen in disease may also translate to the site-level. Also, the lack of site-specific differences in beta diversity seen based on smoking in healthy subjects suggests that smoking contributes to the site-specific differences in disease. Future work should determine how smoking could lead to disparate effects at different sites in the mouth.

Diabetes increases periodontitis risk. The underlying mechanism of this relationship involves immune functioning, neutrophil activity, and cytokine biology[28]. The effects of hyperglycemia on the oral microbiome have been investigated as well. The subgingival environment of a diabetic is glucose-rich, pro-oxidant, protein-rich, and anaerobic. The microbiome of diabetics is comprised of gram-positive facultatives and gram-negative anaerobes[16]. Site-specific differences were only observed for nondiabetic subjects with disease. This could be because the distinct subgingival environment in hyperglycemic individuals promotes a more homogeneous microbiome across sites. This finding disagrees with previous research that found that the dominant species in hyperglycemic individuals varied, which contributes to variation between individuals[16].

While there is a consensus that periodontitis is a site-specific disease, minimal research has focused on discerning the factors that determine the site-specific characteristics of the disease. This study sought to determine if diseased sites in each

individual are different microbially and determine the site-specific factors that contribute to these differences. Since the current understanding of periodontitis is influenced by studies that examined aggregated data from diseased sites, the identification of microbial differences between diseased sites, and the factors contributing to these differences, could transform future periodontal research and treatment. Moreover, the identification of site-specific contributors to disease, such as obesity, hyperglycemia, and smoking status, enable early recognition and interventions for periodontitis, which could have a profound effect on the prevention and control of periodontitis and even, related systemic diseases such as Type-2 Diabetes Mellitus.

In summary, this study highlights the need for the precision of periodontal treatment to be focused on the site-level rather than the patient level because the diseased microbiome is unique to each site.

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